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ELISA to Detect Humoral Antibodies Specific for Clostridium botulinum Type A Neurotoxin

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Short title: ELISA DETECTION OF ANTIBODY TO C. BOTULINUM TYPE A

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

ABSTRACT

The enzyme-linked immunosorbent assay (ELISA) was modified for the rapid detection of human humoral antibodies (IgG) that are specific for Clostridium botulinum type A toxoid. The ELISA was useful for the rapid evaluation of immunization results and for distinguishing (95%) immune from nonimmune individuals. ELISA values, when compared with standard in vivo toxin neutralization test values, paralleled the anamnestic response to booster immunization in 25 of 25 individuals.

Serum antibodies to each of the botulinal neurotoxin types (A-G) are measured by the toxin neutralization (mouse protection) test (3, 12) as modified by the Centers for Disease Control, Atlanta, GA (C. L. Hatheway, personal communication). This in vivo method is based on the mortality of mice during a 4-day observation period, thus precluding rapid diagnoses. A sensitive in vitro test was sought, therefore, to provide rapid detection and identification of humoral antibodies to specific botulinal neurotoxins or neurotoxoids. An in vitro test also would allow nonlethal neurotoxoid to be used as the capture antigen in lieu of active botulinal toxin. Enzyme-linked immunosorbent assays (ELISA) previously have been used to detect botulinal toxin types A, B, E, and G (5, 7, 8, 9). Consequently, it was logical to modify this diagnostic procedure for the identification of antibodies specific for botulinal toxin.

This report describes an indirect ELISA for the rapid detection of human humoral antibodies (IgG) specific for Clostridium botulinum type A toxin and toxoid. The ELISA is compared with the standard toxin neutralization test for measuring these serum antibodies.

MATERIALS AND METHODS

Toxin. C. botulinum type A, Hall strain, was obtained from the Food Research Institute, Madison, WI. A 2-ml quantity of stock culture was inoculated into 100 ml of cooked-meat medium (Difco Laboratories, Detroit, MI). After 24 h of incubation at 37°C, the entire liquid culture was added to 20 liters of steam-sterilized medium consisting of 2% casein hydrolysate (N-Z amine, NAK; Humko Sheffield Chemical Co., Memphis, TN), and 1% yeast extract (Difco), pH 7.3. Filter-sterilized calcium lactate and glucose were added at the time of inoculation to final concentrations of 0.3% and 0.5%, respectively. The culture was incubated statically at 37°C for 5 days; then the toxin and other proteins were precipitated by slowly adjusting the pH of the culture to 4.0 with 3N H_2SO_4. The precipitate was allowed to settle, the fluid removed, and the sediment washed twice with distilled water, then concentrated by centrifugation at 18,000 X g for 15 min. Toxin was extracted from the pellet three times with 200 ml of 0.2M phosphate buffer, pH 6.0, per extraction. The extract was then dialyzed against three changes of 0.05M citrate buffer, pH 5.5, at 4°C for 72 h. The dialysate was centrifuged at 18,000 X g for 15 min, and the supernatant applied through a 2.5 x 40-cm column of microgranular diethylaminoethyl cellulose (DE-52, Whatman, Inc., Clifton, NJ) equilibrated with the previously described citrate buffer. Nonadsorbed proteins, including the A toxin, were eluted with the citrate buffer. Fractions with an absorbance (260/280 nm) ratio of 0.5 to 0.55 representing individual peaks were pooled and dialyzed against three changes of 0.15M Tris buffer (pH 8.0) at 4°C for 72 h.

Samples from the pools were tested for toxicity by the mouse bioassay. The toxin containing pool was centrifuged (18,000 X g for 15 min) and the supernatant added to a 1.5 X 40-cm column of DEAE Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ), which had been equilibrated with the 0.15M Tris buffer (pH 8.0). Adsorbed toxin was eluted with Tris buffer containing 0.1M NaCl. Eluted toxin fractions were pooled and dialyzed against three changes of 0.5 M succinate buffer (pH 5.5). The resulting toxin solution (0.4 mg protein/ml) contained 4.0×10^7 mouse intraperitoneal (i.p.) median lethal doses (LD_{50}) per ml. When 20 µg of this preparation were electrophoresed on 5% sodium dodecyl sulfate-polyacrylamide gels by the method of DasGupta and Sugiyama, (4), only a single protein band representing 150,000 molecular weight was evident. Also, the toxin was shown to be free of hemagglutinin activity. The toxin preparation was divided into two portions. One was used for toxoid production and the other was mixed with an equal volume of glycerol and frozen (-70°) in aliquots.

Toxoid. The purified type A toxin in succinate buffer was converted into toxoid by adding formalin to a final concentration of 0.6%, incubating the mixture at 37°C for 3 days, and then dialyzing it against three changes of the same succinate buffer at 4°C for 72 h. Toxoid (0.5 ml) diluted 1:10 in sterile phosphate gelatin buffer (0.02 M phosphate, 0.2% gelatin, pH 6.2) was injected i.p. into each of four male, 18 to 22 g, Swiss mice. Mice were observed 4 days for signs of botulism and death. They did not develop botulism.

Sera. Sera were selected from each of three groups of frozen (-20°C) human sera. The 52 samples of Group I were selections of blind-coded samples from a collection of sera obtained at random from im-

munized and non-immunized individuals visiting the Clinical Diagnostic Laboratory at our Institute. Group II (75 sera) was composed of sera from individuals that had received 7 to 11 immunizations with Pentavalent (ABCDE) Botulinum Toxoid, aluminum phosphate-adsorbed and/or a monovalent type (A) botulinum toxoid 7 to 8 years ago (6). Subsequently, these individuals were given two booster immunizations 1 year apart, and serum was collected immediately prior to, and at 2 and 15 weeks after, the second booster. Group III sera (7 samples) were from blood drawn from individuals 28 days after the last of three injections with Botulinum Toxoid Adsorbed Monovalent (E). These individuals had no previous history of inoculation with botulinum toxoid (1).

Mouse bioassay. The median lethal dose (LD_{50}) was determined by mouse bioassay using the Reed and Muench method (10). Twofold serial dilutions of toxin were made in sterile gelatin phosphate buffer, a 0.5-ml amount of the diluted toxin was injected i.p. into each of four male Swiss mice, 18 to 22 g, and the mice were observed for 4 days.

Neutralization (mouse protection) method. The in vivo type A toxin neutralization activity of each human serum was determined in International Units (IU) by a mouse protection test. One IU of activity is sufficient to neutralize 10,000 mouse i.p. LD_{50} of type A botulinal toxin. Using a standard toxin challenge, 50% endpoint dilutions of test sera and World Health Organization (WHO) standard type A antitoxin were determined. Comparisons were made with the endpoint dilution of the WHO standard and the test sera. The IU of antitoxin per ml of test serum at the calculated endpoint dilution is equal to that of the antitoxin standard at its endpoint dilution.

ELISA method. An indirect ELISA method for assaying antibodies was used with some modifications (11). The 60 inner wells of disposable polystyrene flatbottomed plates (Microelisa; Dynatech Laboratories, Inc., Arlington, VA) were coated for 60 min at 37°C with 200 µl of a 1:1000 solution of type A botulinal toxoid in 0.1 M Na_2CO_3 buffer (pH 10.6). Unbound toxoid was removed by washing three times with phosphate-buffered saline (pH 7.4) containing 0.05% Triton X-100 (Eastman Organic Chemicals, Rochester, NY). A 200µl aliquot of a 1:100 dilution of the test serum was added and the plate incubated for 90 min at 37°C. The wells were again washed three times and 200 µl of a 1:100 dilution of alkaline phosphatase-conjugated rabbit, anti-human IgG (Microbiological Associates, Bethesda, MD) in phosphate-buffered saline was added. After a 90 min incubation period at 37°C, the excess conjugate was removed by washing each well three times. Two hundred µl of 0.1% disodium p-nitrophenylphosphate (Sigma Chemical Corp., St. Louis, MO) in (pH 10.25) 2-amino-2-methyl-1-propanol buffer (Fisher Scientific, Fair Lawn, NY) warmed to 37°C were added. The enzymatic reaction was stopped by the addition of 50 µl of 3 M NaOH to each well. Optical density of a 250 µl quantity from each well was determined at 400 nm with a Gilford Stasar II (Gilford Instrument Laboratories, Inc., Oberlin, OH) with microsipper attachment. ELISA values were averages of at least three replicate tests; an ELISA value of $\geq$ 0.04 was considered positive. The optical density range for blank and control wells was 0.01 to 0.

All data points for samples obtained 2 and 15 weeks after booster injections were used for analyses. All data points for sera obtained immediately before the second booster were used except that of serum 17

(neutralization titer < 0.1). Correlation and regression analysis were used to evaluate the linear relationship of log (neutralization titer) to ELISA OD (13). Analysis of covariance was used to test the equality of regression lines for weeks 0, 2, and 15.

RESULTS

Forty-five of the 52 Group I sera from individuals having no known history of immunization with botulinum toxid were negative for antitoxin by the in vivo and in vitro tests. Sera from six individuals had neutralizing antibody for type A and antibody detected by ELISA. One additional serum neutralized toxin that was negative by ELISA was from an individual who previously had received three injections of toxoid, each prior to 1960 (Table 1).

Both the ELISA and toxin neutralization methods detected the anamnestic response to booster immunizations with Pentavalent (ABCDE) Botulinum Toxoid (Fig. 1). The decline in serum antitoxin titer between 2 and 15 weeks after booster immunization was documented by both methods. Sera from 4 individuals obtained before the second of the two booster injections had antitoxin by the neutralization test but not by ELISA. The booster immunization did not raise the ELISA value of sera from one of these subjects to a significant level, although the in vivo titer increased from week 0, 0.1 to 0.6, and 1.0 at 2, and 15 weeks respectively.

This ELISA satisfied our requirements for a rapid, sensitive, and economical in vitro screening method. Sensitivity and specificity of the ELISA easily differentiated between immune and nonimmune individuals, in 95% of the 134 serum samples. ELISA values paralleled the anamnestic response to booster immunization in 25 of 25 individuals (Group II). The ELISA was specific for the detection of antibody to type A toxin and gave a negative reaction with sera of individuals immunized with only monovalent type (E) toxoid.

As would be expected, the quantitative results obtained by the in vivo method of measuring toxin neutralizing activity and the in vitro ELISA method of measuring antibody specific for neurotoxoid were not always equivalent nor were the intratest results consistently parallel. For example, an in vivo derived value of 2.6 IU may represent a theoretical range of 2.35 IU to 3.35 IU, without a change in the live/dead ratio of injected test mice. Similarly, the ELISA values corresponding to the nine Group II sera having toxin neutralization values of 2.6 IU/ml ranged in actual values from 0.10 to 0.40. Noncorrelation between mouse toxicity and in vitro tests for the detection of Type A botulinal toxin has been discussed in detail (2). Toxin and antigenic determinants of botulinal toxins are not identical, since nontoxic toxoid is used to induce immunity. In the test serum, only those antibodies which are capable of neutralizing the specific toxin are measured by the in vivo method. The ELISA method, however, will detect all IgG antibodies directed against the neurotoxoid. Significant linear correlations were found between log (neutralization titer) and ELISA OD before and after booster injection (Figs. 2, 3). This allows one to be confident in relating specific neutralization titers to ELISA values. For example, from Fig. 3 we see that a neutralization titer of 10 IU ($\log_{10} = 1.00$) corresponds to an average ELISA OD (95% confidence limits) of 0.52 ± 0.03.

In only 5% (7 of 134) of the sera tested was toxin neutralizing activity detected and a negative ELISA obtained for the same sample. More importantly, no false positives were seen in the ELISA method, when compared with in vivo results. In the ELISA method sera were tested at a 1:100 dilution which allowed for a minimal ($\geq$0.02) background OD from known negative sera. However, this use of diluted sera is thought to have contributed to the reduced ($\approx$0.5 IU/ml) ELISA sensitivity. The toxin neutralization method can accommodate a 1:2 dilution of test sera and has a sensitivity of 0.02 IU/ml.

The ELISA method described is most satisfactory for the rapid evaluation of immunization results, for monitoring titer changes of immunized at-risk laboratory workers, and for distinguishing immune from nonimmune individuals.

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TABLE 1. Immunization histories and ELISA values of the 7 group I sera that neutralized Clostridium botulinum type A toxin

Serum no.	Immunization history[a]	Toxin neutralization[b]	ELISA
8	5, pre-1955	0.50	0.04 ± 0.01
11	3, pre-1960	0.50	0.03 ± 0.01
13	10, pre-1970	32.80	0.94 ± 0.12
19	3, in 1980	1.40	0.13 ± 0.01
24	11, pre-1960	0.60	0.25 ± 0.02
25	12, pre-1960	1.80	0.11 ± 0.01
47	4, 1977-80	0.60	0.21 ± 0.02

[a] Number of immunizations with Pentavalent (ABCDE) Botulinum Toxoid and/or Monovalent (A) Botulinum Toxoid.

[b] Standard in vivo (mouse protection) test, values expressed in IU/ml serum.

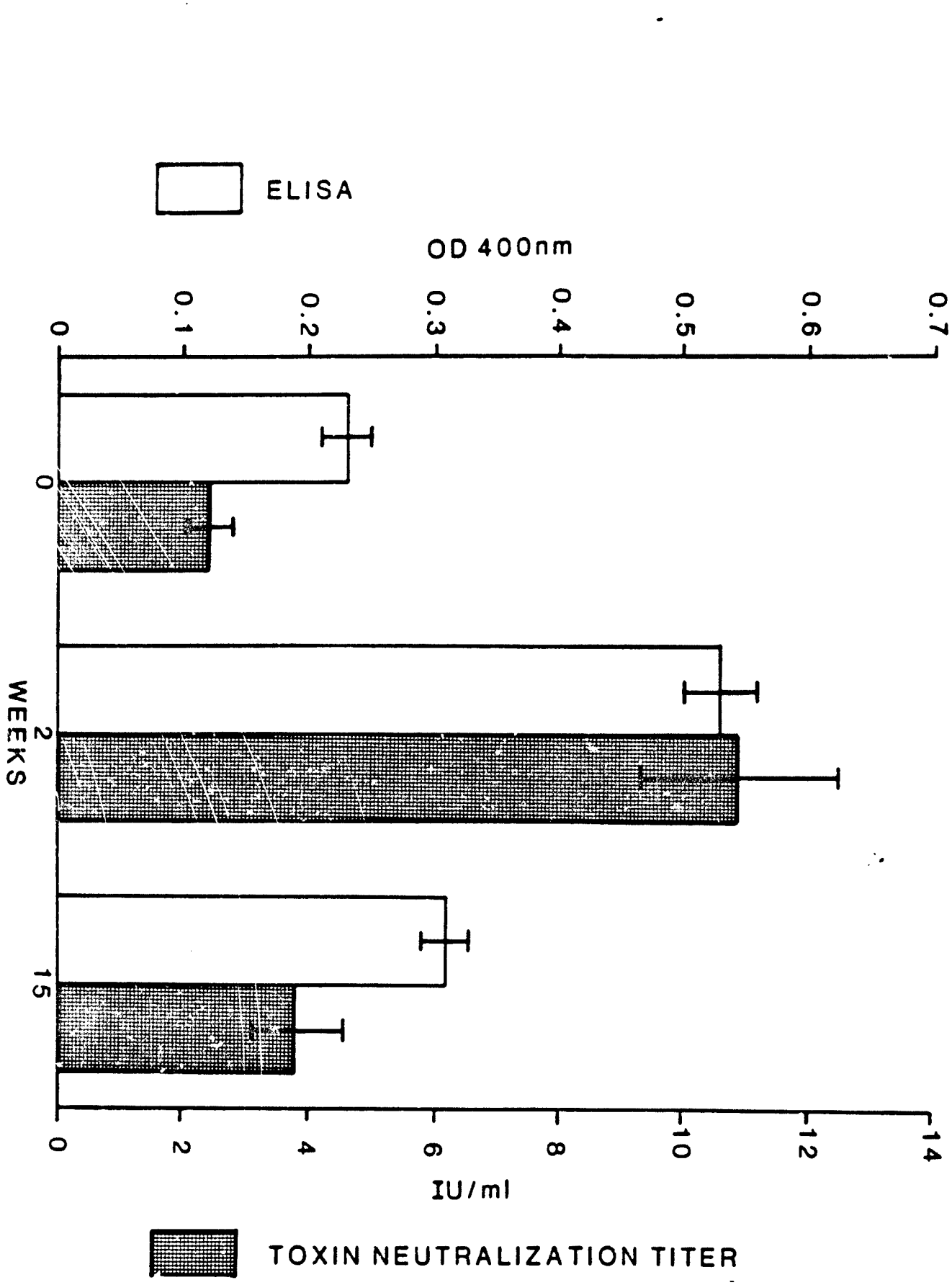
[c] Optical density, 400 nm ± SE. Neg. < 0.04, Pos. ≥ 0.04.

FIGURE LEGENDS

FIG. 1. Comparison of the ELISA and toxin neutralization methods for measurement of the anamnestic response of 25 individuals to booster immunization with Botulinum Toxoid (mean + standard error), following a lapse of 7 to 8 years. Values are for sera collected before the second of two booster doses and at 2 and 15 weeks thereafter.

FIG. 2. Linear regression and 95% confidence limits (dashed line) for predicting an average ELISA OD for a given value of log (neutralization titer) from sera collected 12 months after initial booster injection. $r^2 = 0.452$, $p < .001$.

FIG. 3. Linear regression and 95% confidence limits (dasned line) for predicting an average ELISA OD for a given value of log (neutralization titer) at 2 and 15 weeks (combined) after second booster injection $r^2 = 0.766$, $p < .001$.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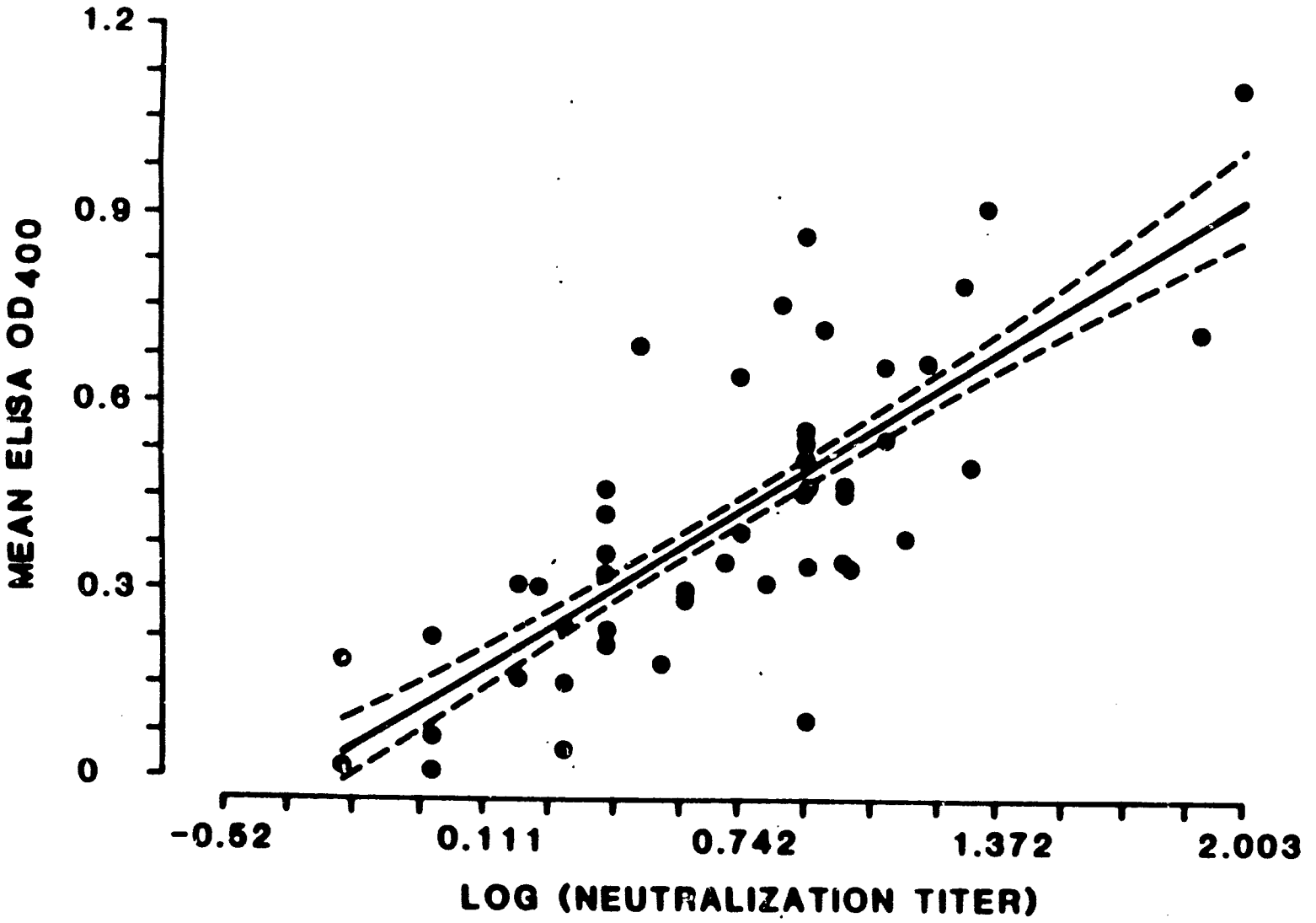
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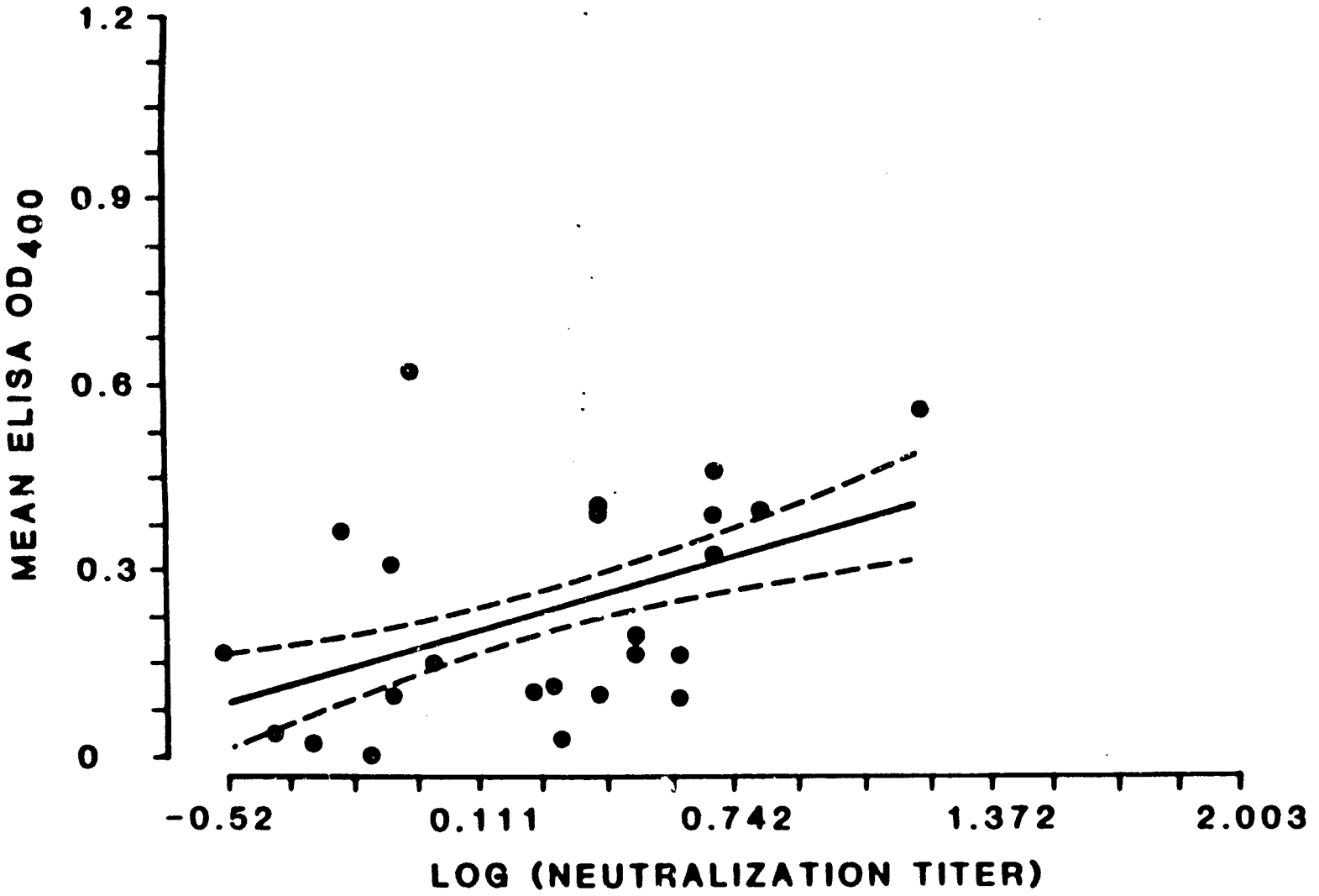
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